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Quantitative relationships between induced jasmonic acid levels and volatile emission in *Zea mays* during *Spodoptera exigua* herbivory

Abstract Jasmonic acid (JA) has long been hypothesized to be an important regulator of insect-induced volatile emission; however, current models are based primarily on circumstantial evidence derived from pharmacological studies. Using beet armyworm caterpillars (BAW: *Spodoptera exigua*) and intact corn seedlings, we examine this hypothesis by measuring both the time-course of insect-induced JA levels and the relationships between endogenous JA levels, ethylene, indole and sesquiterpenes. In separate Morning and Evening time-course trials, BAW feeding stimulated increases in JA levels within the first 4–6 h and resulted in maximal increases in JA, indole, sesquiterpenes and ethylene 8–16 h later. During BAW herbivory, increases in JA either paralleled or preceded the increases in indole, sesquiterpenes and ethylene in the Morning and Evening trials, respectively. By varying the intensity of the BAW herbivory, we demonstrate that strong positive relationships exist between the resulting variation in insect-induced JA levels and volatile emissions such as indole and the sesquiterpenes. To address potential signaling interactions between herbivore-induced JA and ethylene, plants were pretreated with 1-methylcyclopropene (1-MCP), an inhibitor of ethylene perception. 1-MCP pretreatment resulted in reduced production of ethylene and volatile emission following BAW herbivory but did not alter the insect-induced accumulation of JA. Our results strongly support a role for JA in the regulation of insect-induced volatile emission but also suggest that ethylene perception regulates the magnitude of volatile emission during herbivory.

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Introduction

Plants display a diverse array of inducible changes in secondary metabolism following mechanical and biotic stresses (Karban and Baldwin 1997). In tomato leaves, protease inhibitors are rapidly induced following both mechanical damage and insect herbivory (Green and Ryan 1972). These direct defenses are known to reduce insect growth rates by interfering with the digestibility and nutritive quality of plant tissues (Johnson et al. 1989). Plants also utilize indirect defenses; for example, induced volatiles triggered by insect feeding serve as attractants for predators and parasitoids searching for their respective prey and hosts (Turlings et al. 1990; Vet and Dicke 1992). In corn (*Zea mays*) seedlings, only low levels of volatiles are released following mechanical damage (Turlings et al. 1990). However, only a few hours after armyworm (*Spodoptera* sp.) herbivory, indole and a range of sesquiterpenes become dominant headspace components (Turlings et al. 1991; Gouinguené et al. 2001). Beet armyworm (BAW; *Spodoptera exigua*) oral secretions (OS), applied to mechanical damage sites, are sufficient to mimic the plant response to caterpillar feeding (Turlings et al.

1990, 1993). Volicitin [*N*-(17-hydroxylinolenoyl)-L-glu-tamine], a fatty acid-amino acid conjugate, has been isolated and identified in BAW OS and acts as potent elicitor of volatile emission in excised corn seedling bioassays (Alborn et al. 1997; Schmelz et al. 2001).

Volicitin was the first non-enzymatic elicitor of vola-tile biosynthesis isolated from an insect herbivore. Ad-ditional related fatty acid-amino acid conjugates with biological activity have now been identified in the OS of several Lepidoptera species (Paré et al. 1998; Pohnert et al. 1999; Alborn et al. 2000; Halitschke et al. 2001). Recent progress on the regulation of volatile biosynthetic pathways in corn supports the role of insect-derived elicitors in mediating the differential responses following mechanical damage and insect herbivory. Frey et al. (2000) demonstrated that volicitin rapidly triggers indole production by selectively increasing the transcription levels of indole-3-glycerol phosphate lyase (IGL). IGL channels indole-3-glycerol phosphate to volatile indole production instead of alternatives such as tryptophan or non-volatile defenses such as hydroxamic acids. Simi-larly, Shen et al. (2000) established that volicitin signifi-cantly increased mRNA levels of a sesquiterpene cyclase (*stc*1) and production of a putative sesquiterpene volatile in corn. Additional enzymes in corn foliage such as (*E*)-nerolidol synthase, a key intermediate in the synthesis of the volatile (*3E*)-4,8-dimethyl-1,3,7-nonatriene, are induced following *Spodoptera* herbivory (Degenhardt and Gershenzon 2000).

Despite these recent advances in understanding elic-itor and insect-induced changes in volatile biosynthetic pathways, the insect-induced signaling cascades involved in volatile emission are still unclear. Jasmonic acid (JA) has long been considered as a candidate endogenous regulator as exogenous applications of JA promote volatile emission in numerous excised-leaf bioassays (Hopke et al. 1994; Boland et al. 1995). This hypothesis is supported as caterpillar herbivory is known to result in rapid increases in JA levels (McCloud and Baldwin 1997). More recently, Halitschke et al. (2001) demon-strated that a combination of fatty acid-amino acid conjugates present in *Manduca sexta* OS are sufficient to increase both JA levels and volatile emission in wild tobacco (*Nicotiana attenuata*). While these results col-lectively suggest that insect feeding damage increases JA levels and ultimately volatile emission, the existence of quantitative endogenous relationships between herbi-vore-induced JA levels and volatile emission has yet to be demonstrated in any system.

In addition to JA, herbivory stimulates the produc-tion of ethylene (Kendall and Bjostad 1990; Kahl et al. 2000). Signaling interactions between JA and ethylene have been demonstrated to result in either synergistic (Xu et al. 1994; O'Donnell et al. 1996; Penninckx et al. 1998) or antagonistic interactions (Zhu-Salzman et al. 1998; Winz and Baldwin 2001) in the expression of plant defense responses to pathogens and insects. In the case of volatile emission in plants, a role for ethylene remains unclear. Using detached lima bean leaves (*Phaseolus*

lunatus), Horiuchi et al. (2001) demonstrated that exogenous applications of the ethylene precursor, 1-am-inocyclopropane-1-carboxylic acid, enhances JA-induced volatile emission. However, in tobacco (*N.attenuata*), Kahl et al. (2000) did not detect any significant interac-tions between exogenous methyl jasmonate, ethylene or ethephon (an ethylene releaser) upon induced emission of the sesquiterpene, bergamotene.

At the plant-insect interface, caterpillar feeding is known to result in mechanical damage and quite likely the presence of OS, elicitors and saliva on the plant leaf sur-face (Alborn et al. 1997; Felton and Eichenseer 1999; Halitschke et al. 2001). These combined stimuli represent the plant's perception of insect attack and together ulti-mately trigger plant defense responses such as induced volatile emission. A better understanding of insect-in-duced plant signaling and hormonal changes is required if these plant responses are to be beneficially manipulated. To date, very few studies have rigorously quantified the time-course of plant hormone changes and plant defense responses during actual insect herbivory (however, see McCloud and Baldwin 1997). As an important baseline in understanding herbivore-induced signaling events leading to volatile emission in corn, we examined (1) the effect of BAW feeding on JA levels and volatile emission, including ethylene, indole and sesquiterpenes. Two different time-courses, denoted as Morning and Evening, were per-formed to examine temporal associations between signals and responses. We then determined (2) if quantitative relationships exist between the level of BAW herbivory, JA and resulting volatile release and (3) we used 1-meth-ylcyclopropene (1-MCP), an inhibitor of ethylene per-ception, to examine potential interactions between ethylene, herbivore-induced JA and volatile emission.

Materials and methods

Plant growth and insect rearing

Seeds of *Z. mays* L. cv. Delprim were acquired from Delley Seeds and Plants (Delley, Switzerland) and germinated in potting soil (MG500SC; Scotts-Sierra Horticultural Products, Marysville, Ohio). After 6 days of growth, seedlings were removed from soil, the roots rinsed with water, and transferred to individual 1 l hy-droponic containers. Plant nutrient additions follow Schmelz et al. (2001). All plants were maintained in a 12 h photoperiod (6 a.m. to 6 p.m.) with 350 μmol m^{-2} s^{-1} of photosynthetically active radia-tion, 70% relative humidity and a temperature cycle of 22°C/26°C (night/day). Experiments utilized 10-day-old plants carrying three leaves. BAW larvae (*S. exigua*) were obtained from Dr. W. J. Lewis (IBPMRL, USDA-ARS, Tifton, Ga.) and reared on an artificial pinto bean diet, following the method of King and Leppla (1984). Early third instar larvae were selected for all insect experiments.

Experimental designs

Immediately prior to the start of caterpillar infestation experiments, hydroponically grown intact plants were placed into chambers con-sisting of long glass cylinders (61 cm ×17 mm i.d.). The straight, yet laterally confined, leaves occupied the upper two-thirds of the cylinders while the out-stretched roots filled the lower one-third. The

bottom portions (lower one-third) of the chambers were then placed back into the hydroponic containers. Thus, roots were returned to their original nutrient solutions, and the leaves remained in a dry and upright position. At the beginning of each experiment, BAW caterpillars were placed in the glass chambers and allowed to roam freely throughout the plant foliage. A cotton plug provided a barrier at the root-shoot interface to prevent caterpillar access to the roots and hydroponic solution. The glass chambers provided four experimental advantages. Firstly, caterpillars with a propensity for wandering were confined largely to the plant leaves, thus generating more uniform levels of herbivory between replicates. Secondly, the chambers could be briefly sealed for estimates of intact-plant ethylene production. Third, collection of plant volatiles during herbivory was enabled without further mechanical perturbation of the plants or insects. Fourth, analysis of hormones and logistics of volatile emission was possible from the same plants, avoiding the need for simultaneous parallel experiments.

Insect infestation experiments

To investigate the time-course of insect-induced plant hormone fluctuations and volatile emission we utilized two starting points, 6 a.m. and 6 p.m., designated as Morning and Evening trials, respectively. The Morning and Evening trials were initiated by placing seven BAW larvae in each plant chamber at either the start of the photophase (6 a.m.) or scotophase (6 p.m.), respectively. Control groups for each time received neither caterpillars nor additional wounding. All experiments utilized four replicates per treatment group and required destructive harvesting of plants at each time point for analysis. The Morning and Evening trials were successively sampled for ethylene, volatiles and JA at either at 0, 4, 8, 12 h or 0, 6, 11, 13, 18 h after initiation of the experiment, respectively, and required a total of 28 or 36 plants. Relationships between caterpillar infestation level, plant hormones, and volatile emission were investigated in plants ($n = 4$) infested with 0, 1, 2, 4, 6, or 8 BAW larvae at the beginning of the scotophase (6 p.m.). Ethylene, volatile emission, and JA samples were collected at one time point, between 8 and 10 a.m., in the following photoperiod.

To investigate the role of herbivore-induced ethylene, intact plants ($n = 32$) were incubated in individual sealed 7 l Plexiglas cylinders (12 cm ×62 cm) for 12 h (6 p.m.–6 a.m.) in the presence of air ($n = 16$) or air containing 15 µl l^{-1} 1-MCP gas; referred to as air pretreatment and 1-MCP pretreatment, respectively. Pure 1-MCP gas was generated by dissolving EthylBloc (0.43% 1-MCP, Bio-Technologies for Horticulture, Walterboro, S.C.) over a column of EthylBloc-releasing buffer and subsequently collected with airtight syringes. At the beginning of the photophase (6 a.m.), all plants were removed from their individual chambers and placed into glass chambers for experimentation. The eight treatment groups consisted of both uninfested controls ($n = 4$) and BAW infested plants ($n = 4$) overlying four additional treatment combinations: (1) air pretreatment, (2) 1-MCP pretreatment, (3) air pretreatment plus ethylene, and (4) 1-MCP pretreatment plus ethylene. Six BAW caterpillars were utilized to infest each designated plant and the experiment was initiated at 8 a.m. All glass chambers were internally rigged with microbore PTFE tubing that terminated at the root/shoot interface. Using this tubing, the individually regulated flow (5 ml min^{-1}) of air (treatments 1, 2) and air containing ethylene (treatments 3, 4; 500 ppb ethylene; BOC Gases, Riverton, N.J.) was initiated at 10 a.m. and continued for 5 h until 3 p.m. Exogenous additions of ethylene at this concentration synergize the volatile-inducing activity of purified insect elicitors, such as volicitin (E.A. Schmelz et al. unpublished data). At 3 p.m. all glass cylinders were flushed with 5 l of compressed air and sealed for the subsequent collection of plant-produced ethylene, volatiles, and JA analysis.

Coordination of analyses

Ethylene production, volatile emission and JA levels were sampled from each plant. Prior to the volatile collection period, rubber septa (#37 SubaSeal, Aldrich) were used to seal both ends of the glass cylinders for 1 h. At this time a 1 ml headspace sample was withdrawn and the septa were replaced with those adapted for volatile collection. Immediately thereafter, volatiles were collected for 30 min with the BAW larvae still present in the chambers. Upon completion of volatile collection, plants were quickly removed from the chambers, cleaned of caterpillars and frass, weighed, and snap frozen in liquid nitrogen for JA analysis.

Ethylene analysis

Ethylene production from intact plants was determined by removing 1 ml of headspace from plant chambers temporarily sealed for 1 h. The headspace sample was analyzed by gas chromatography (GC) on a Hewlett-Packard (HP)-5890 GC with injector, oven and flame ionization detector temperatures of 150, 80, and 250°C, respectively, and a HayeSep Q column (80/100 mesh, 6 feet ×0.125 inches ×0.085 inches i.d., Alltech, Deerfield, Ill.) using a nitrogen carrier gas flow rate of 100 ml min^{-1}. Quantification was based on an external standard curve constructed from 1 ml injections of known ethylene standards (Kao and Yang 1983). Calculations of ethylene production (nl g^{-1} h^{-1}) were based on the wet mass of the whole plant as headspace samples were removed from intact plants consisting of both roots and shoots.

Volatile analysis

Intact plants were placed into glass vessels and assayed under their original lighting conditions. Experiments utilized glass cylinders (17 mm i.d. ×61 cm long, 127 ml volume) and collection of volatiles followed Turlings et al. (1991). Briefly, clean humidified air was passed through the vessels (550 ml min^{-1}) and volatiles were trapped on 30 mg Super Q (80/100 mesh; Alltech). The super Q traps were eluted with 150 µl dichloromethane and 400 ng nonyl acetate (in 5 µl dichloromethane) was added as an internal standard. Quantification of volatiles was performed on an HP 6890 GC according to Schmelz et al. (2001). Our use of the term 'combined sesquiterpenes' refers only to the summation of β-caryophyllene, (E)-α-bergamotene, and (E)-β-farnesene levels.

JA quantification

The extraction and quantification of JA was modified from Weber et al. (1997). At specified times, leaf tissues were excised from intact plants, snap frozen in liquid N_2 and ground to a fine powder using a mortar and pestle. Weighed samples of approximately 1 g were extracted in 3.5 ml methanol with 500 ng of the internal standard dihydrojasmonic acid (dhJA). Methyl-dhJA (Bedoukian Research, Danbury, Conn.) was subjected to alkaline hydrolysis to yield dhJA. The absence of dhJA in control and induced corn tissues was first confirmed prior to its use as an internal standard. After 30 min in a sonicating bath, each sample was mixed with 1.5 ml water, shaken and centrifuged at 12,000 rpm for 5 min. The resulting supernatant was saved, adjusted to pH 8.5 with aqueous 1 M NH_4OH and kept on ice. Solid phase extraction (SPE) cartridges (Reverse Phase C18, 12 ml, Mallinckrodt Baker, Griesheim, Germany) were washed first with 8 ml each of methanol, then 7:3 methanol:H_2O. Each sample was passed through a column followed by 7 ml 75:25 methanol:H_2O. All eluate was collected, brought to pH 3.5 with 10% formic acid, and raised to a total volume of 50 ml with H_2O. The used SPE cartridges were cleaned and conditioned with 5 ml each successively of methanol:formic acid (99.2:0.8), methanol, diethylether, methanol, and then 10 ml H_2O. The samples were then reloaded on the columns and washed with 7 ml each of ethanol:H_2O (15:85) and H_2O. The oxylipin fraction was eluted with 10 ml diethylether, and the remaining ether layer was dried under N_2, transferred to a 2 ml reaction vial where methanolysis was accomplished by adding 30 µl of HCl:methanol (1:2) for 12 h at 30°C. The HCl:methanol was then

removed under N_2 gas stream and, immediately upon the visual disappearance of the liquid, each sample brought up to 75 μl in methyl-Cl_2. Samples were analyzed by GC-mass spectrometry (MS) on an HP-6890 GC (He carrier gas; 0.7 ml min^{-1}; splitless injector 240°C, injection volume 2 μl) with an HP-5MS column (5% phenyl methyl siloxane, 30 m ×250 μm i.d. ×0.25 μm film thickness) with the temperature programmed from 40°C (1 min hold) at 10°C min^{-1} to 240°C (hold for 15 min). The GC was coupled to an HP 5973 quadrapole-type mass selective detector with transfer line, source, and quadrapole temperatures of 230°C, 230°C and 150°C, respectively. Chemical ionization with isobutane as the reaction gas generated predominantly M + 1 parent ions scanned at a range of 60–500 amu. Retention times and M + 1 diagnostic ions of the JA and dhJA methyl esters were 15.88 min (M + 1 = 225) and 15.98 min (M + 1 = 227), respectively. In a replicated (n = 24) standard addition experiment with JA, recovery was 44% and the reproducibility of recovery (coefficients of variation, n = 6) was 21%. These values are in accordance with Weber et al. (1997). Standard curves, constructed from independent weighings (range 0.1–4 ng μl^{-1} in methyl-Cl_2) of JA and dhJA methyl esters resulted in linear M + 1 responses of 225 and 227 (both r^2 = 0.99) with identical slopes. Diagnostic ion intensity between the methyl esters of JA and the dhJA internal standard were the same, thus use of response factors was not required.

Statistics

Analyses of variance (ANOVAs) were performed on the JA pools, ethylene production and volatiles. Significant treatment effects were investigated when the main effects of the ANOVA were significant ($P < 0.05$). Where appropriate, Tukey tests were used to correct for multiple comparisons between untreated controls and treatments over time. Prior to statistical analysis, all data were subjected to square root transformation to compensate for elevated variation associated with larger mean values (Zar 1996). The analysis was accomplished with JMP 3.0 statistical discovery software (SAS Institute, Cary, N.C.). Percent maximum relative increase of JA and volatiles was estimated by calculating fold increases based on average BAW-induced levels and control starting levels. These fold increases were then divided by the maximum fold increase found to occur at any time point in the experiment.

Results

Kinetics of herbivore-induced JA, ethylene and volatiles

JA and ethylene

Caterpillar feeding rapidly induced the accumulation of JA, and this response preceded or paralleled the induced emission of plant volatiles in the Evening and Morning trials, respectively. Independent of the photoperiod, BAW herbivory stimulated similar increases in foliar JA levels within the first 4–6 h (Fig. 1A). Likewise, both trials resulted in maximal JA concentrations of 72–75 ng g^{-1}, which occurred 8–13 h after the initiation of continuous feeding damage (Fig. 1A). In the Morning trial, BAW-induced JA levels paralleled increases in volatile emission while in the Evening trial, increases in JA levels preceded significant volatile emission. Baseline levels of JA in undamaged control plants were low and ranged from 0.7 to 10.4 ng g^{-1} over time and between experiments (Fig. 1A). Unlike JA, ethylene levels displayed no significant increases during the first 4–6 h of
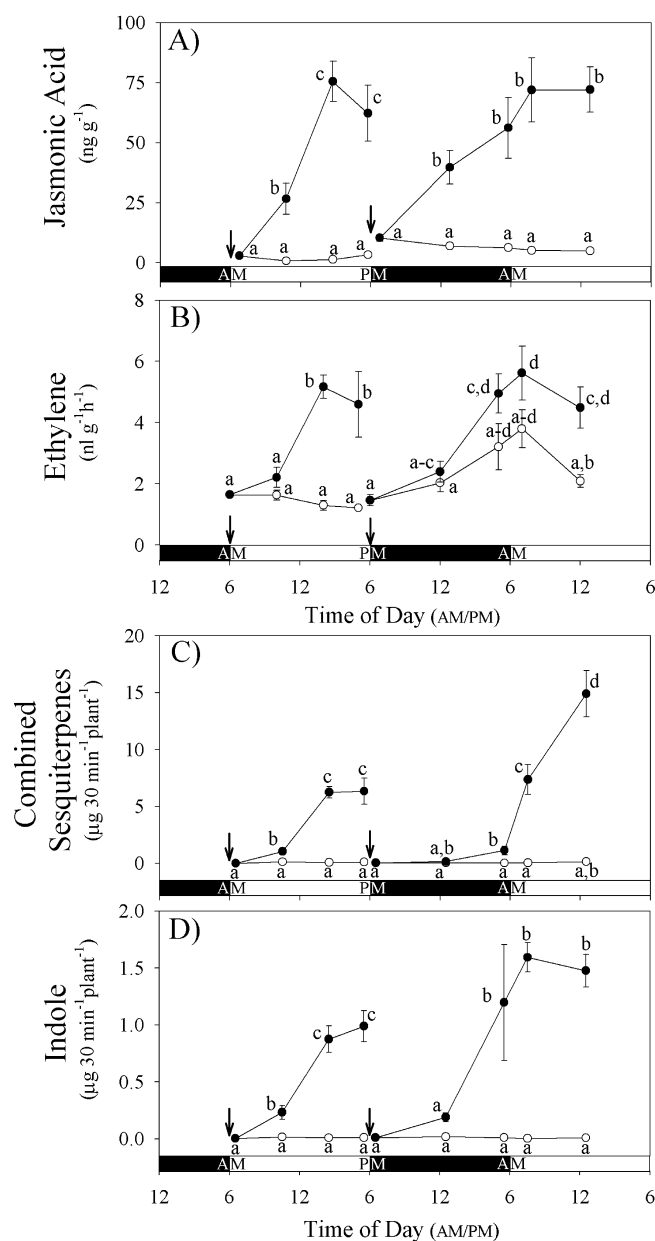

Fig. 1 Mean (±SE) jasmonic acid (JA) concentrations (**A**), ethylene production (**B**), combined sesquiterpene (**C**) and indole emission (**D**) from untreated control (*open circles*) corn seedlings or those infested with beet armyworm (BAW) caterpillars (*closed circles*) at times denoted by *downward arrows*. *White* and *black bars* along the *x*-axis represent the light (photophase) and dark (scotophase) cycles, respectively. Trials initiated at the beginning of the photophase and scotophase are referred to as Morning and Evening, respectively. Within each trial, symbols not sharing the same letters (*a, b, c, d*) represent significant differences ($P < 0.05$, Tukey correction for multiple comparisons)

BAW herbivory (Fig. 1B). A maximal 4.0-fold increase in ethylene production (5.2 nl g^{-1} h^{-1}) was observed in the Morning trial after 8 h, while in the Evening trial a significant increase in ethylene was not detected until 18 h later during the following photophase (Fig. 1B). In the Evening trial, average ethylene production from control plants was also elevated at night and the early

morning; however, this trend was not statistically significant.

Coordination of volatile emission

In the Morning trial, induction of sesquiterpene volatile release was detected within the first 4 h of herbivory, with emission increasing at 8 h and remaining unchanged at 11 h. In the Evening trial, sesquiterpene volatile release was delayed and was not detected until 11 h into the scotophase followed by a rapid increase in emission after the initiation of the photophase. Unlike the Morning trial, sesquiterpene emission did not plateau at 6–7 µg 30 min^{-1} plant^{-1} but instead increased to 15 µg 30 min^{-1} plant^{-1} within 6 h of the beginning of the photophase. In the Morning trial, the release of volatile indole displayed an induction kinetic similar to the sesquiterpenes. Increases were first evident after 4 h and maximal release appeared to stabilize between 8 and 11 h. In contrast, near maximal indole release in the Evening trial was detected at the end of the scotophase and remained stable at 1.5–1.6 µg 30 min^{-1} plant^{-1} throughout the photophase. This suggests that carbon from current photosynthesis is not an absolute requirement for significant indole release. As an estimate of the early coordination of induction in the Morning timecourse trial at 4 h, JA levels, combined sesquiterpenes and indole emission corresponded to 35%, 16% and 23% of their maximum relative increase, respectively. A similar 6 h estimate of the coordination of induction in the Evening time-course trial reveals that JA levels, combined sesquiterpenes and indole emission corresponded to 48%, 0% and 11% of their maximum relative increase, respectively. A preferential increase in JA in the early stages of herbivory is expected if JA is to function as signal for volatile induction.

Relationships between caterpillar infestation levels, JA and volatiles

By varying the intensity of the stimulus, namely the caterpillar infestation level, we demonstrate that direct positive relationships exist between the variation in endogenous JA and volatile emission. BAW infestation levels of two, four or six caterpillars per plant resulted in the induction of foliar JA levels corresponding to 31, 61 and 107 ng g^{-1}, respectively (Fig. 2A). On average, herbivory by a single BAW larva resulted in a 12-fold increase in JA levels (Fig. 2A). Unlike JA, changes in ethylene production were not as dramatic at lower levels of BAW infestation. Herbivory by either one or two BAW resulted in greater mean levels of ethylene production; however, significant increases were only detected at four or more BAW larvae per plant (Fig. 2B). An infestation of six BAW per plant resulted in a 4.2-fold increase in ethylene production compared to the uninfested control plants (Fig. 2B). Trends for
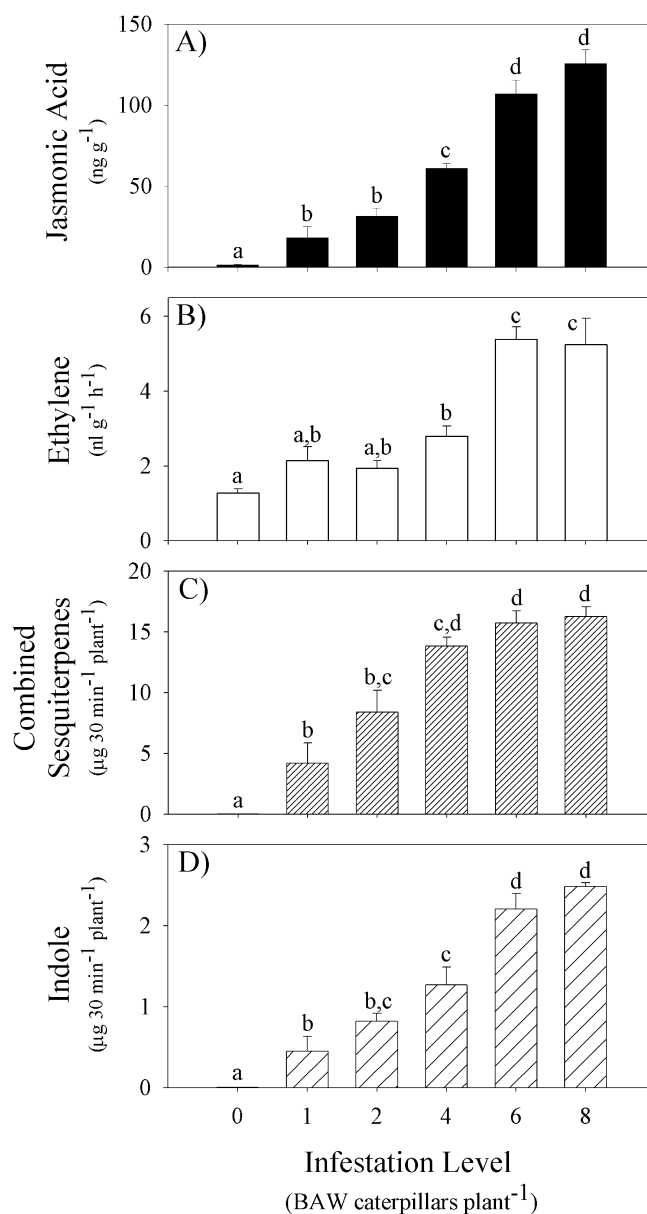

Fig. 2 Mean (+SE) JA concentration (**A**), ethylene production (**B**), combined sesquiterpene emission (**C**) and indole emission (**D**) from intact corn seedlings subjected to a range of BAW infestation levels (0–8 larvae plant^{-1}). Different letters (*a*, *b*, *c*, *d*) represent significant differences within each graph ($P > 0.05$, Tukey correction for multiple comparisons)

volatiles largely matched those of JA levels with combined sesquiterpene and indole emission significantly increasing up to the four and six BAW plant^{-1} infestation level, respectively (Fig. 2C, D). Both indole and sesquiterpene volatile emission demonstrated strong positive relationships with the JA levels induced by BAW feeding (Fig. 3A, B). The increase in combined sesquiterpene release and corresponding JA levels best followed a second order polynomial (ANOVA, $F_{2,21}$ = 89.2, $P < 0.0001$, $r^2 = 0.895$) (Fig. 3A). This indicates that BAW-induced JA levels above 60 ng g^{-1} wet mass do not correspond with greater increases in sesquiter-

pene emission. In contrast to the sesquiterpenes, indole release exhibited a strong linear relationship with JA levels across the entire range of BAW infestation levels examined (ANOVA, $F_{1,22} = 233.6$, $P < 0.0001$, $r^2 = 0.914$) (Fig. 3B).

1-MCP reduces ethylene production and volatiles but not herbivore-induced JA levels

Pretreatment of plants with 1-MCP, an ethylene perception inhibitor, and subsequent infestation with BAW larvae had no significant effect on JA levels but significantly reduced herbivore-induced ethylene production, combined sesquiterpene and indole emission. The addition of ethylene to plants pretreated with 1-MCP could not recover the 1-MCP inhibition response, indicating that 1-MCP acts largely through the inhibition of ethylene perception even though production is also affected. Between BAW herbivory treatments, average JA levels ranged from 54 ng g^{-1} to 66 ng g^{-1} and did not differ significantly between groups (Fig. 4A). Unlike JA, BAW-induced ethylene production declined by 2-fold in plants pretreated with 1-MCP (Fig. 4B). Plants pre-

treated with 1-MCP also demonstrated 2-fold decreases in the level of combined sesquiterpenes emitted (Fig. 4C). 1-MCP pretreatment dramatically reduced the level of BAW-induced indole emission by greater
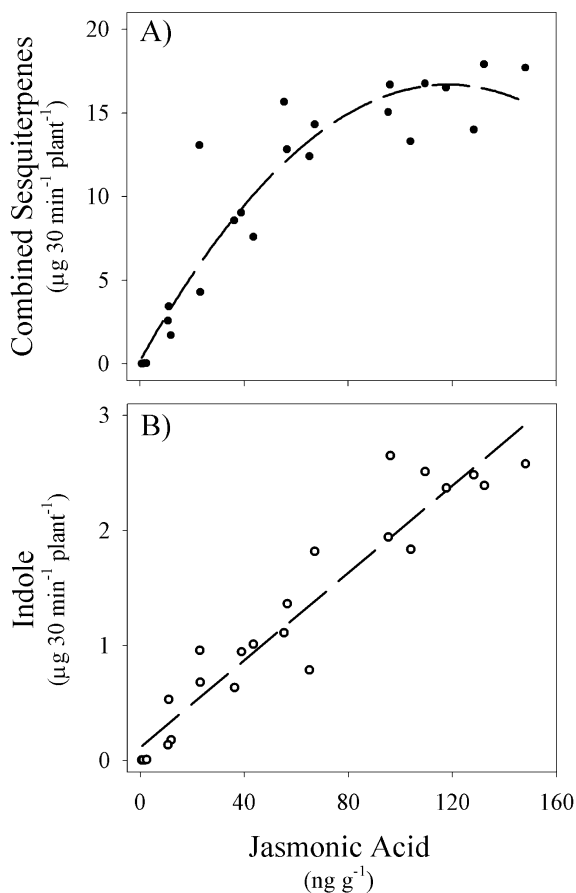

Fig. 4 Mean ($+$SE) JA concentrations (**A**), ethylene production (**B**), combined sesquiterpene (**C**) and indole (**D**) emission from intact corn seedlings pretreated overnight (6 p.m. to 6 a.m.) with either air (*Control, E*) or 15 μl l^{-1} of 1-methylcyclopropene (*1-MCP, 1-MCP + E*). Plants were assigned to either uninfested control groups (**C**) or infested with six early third-instar BAW early in the photoperiod (8 a.m.). Between 10 a.m. and 3 p.m., all plants had 5 ml min^{-1} air flow through the individual glass chambers. Treatments denoted as *Control* and *1-MCP* received regular air while the ethylene addition groups (*E, 1-MCP + E*) received air containing 500 ppb ethylene. Immediately prior to the initiation of hormone and volatile analysis all plant chambers were flushed with clean air. Different letters (*a, b, c*) represent significant differences within each graph (*P* > 0.05, Tukey correction for multiple comparisons)

Fig. 3 Relationships between JA concentration, combined sesquiterpenes (**A**) and indole (**B**) emission stimulated by a range of BAW infestation levels (0–8 larvae plant^{-1})
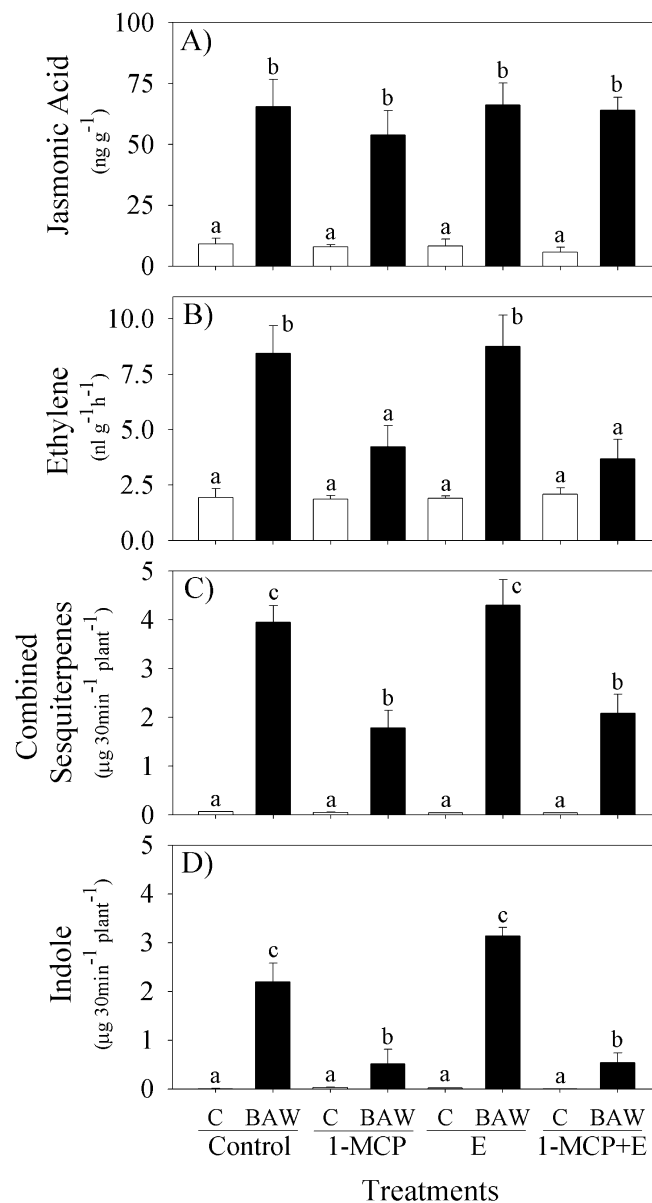
than 4-fold (Fig. 4D). In each case, additions of ethylene during BAW herbivory did not influence the levels of induced JA, ethylene production, sesquiterpenes, or indole compared to BAW-infested plants treated with air only or 1-MCP gas. Thus, the effect of 1-MCP on volatile emission is due to an inhibition of ethylene perception and not to changes in actual ethylene production. Observations of insect feeding and leaf area removed suggested no differences between control and 1-MCP-treated plants. As a precaution to account for possible differences in insect behavior triggered directly or indirectly by 1-MCP, similar experiments were performed using controlled levels of mechanical damage and insect elicitors applied to leaves. In confirmation, identical patterns of unchanged JA induction and reduced volatile emissions were obtained (E.A. Schmelz et al. unpublished data).

Discussion

We have demonstrated that BAW herbivory on corn seedlings induces foliar JA levels and volatiles such as ethylene, sesquiterpene and indole. The induced accumulation of JA was independent of the light cycle and paralleled or preceded volatile emission in separate Morning and Evening time course trials, respectively. Over a range of BAW infestation levels, increasing caterpillar herbivory resulted in stepwise increases in foliar JA levels and total plant volatile emissions. By varying the level of stimulus, we show for the first time that the resulting variation in herbivore-induced JA levels exhibit strong positive relationships with both indole and sesquiterpene emission. Ethylene production also increased with BAW herbivory levels but less uniformly. By pretreating plants with 1-MCP and inhibiting ethylene action, we demonstrate a significant reduction in insect-induced volatiles but no alteration in typical insect-induced JA levels. This suggests that both JA and ethylene perception have significant roles in the regulation of insect-induced volatile emission.

During the photoperiod, induced volatile emission in response to BAW herbivory is rapid. Both indole and the combined sesquiterpenes demonstrated significant increases within the first 4 h when BAW feeding was initiated at the beginning of the light cycle. Not surprisingly, these volatile responses were delayed in the Evening trial when herbivory was initiated at the beginning of the scotophase. In cotton, insect-induced volatiles are known to be synthesized de novo during photosynthesis (Paré and Tumlinson 1997). In both herbivory time-course trials, sesquiterpene volatile emission continued to increase in the 4–8 h following the start of the photophase. These volatile release kinetics are very similar to those found by Turlings et al. (1998) when *Spodoptera littoralis* OS was applied to wounded corn leaves at the beginning of the photophase. Induced emission of indole stimulated by caterpillar OS or purified volicitin is also recognized as a very rapid response (Turlings et al. 1998; Frey et al. 2000).

In corn seedlings, volicitin induces increases in accumulation of mRNA encoding IGL within 1 h and acts to liberate free indole within 1.5 h (Frey et al. 2000). What has not been previously appreciated is that indole can reach near maximal emission levels during nocturnal herbivory. Given this photoperiodic independence, it is possible that indole could function ecologically as an early morning signal for parasitoids and predators searching for insect hosts and prey (Turlings et al. 1991). Unlike the biosynthetic pathways, endogenous signals that regulate indole volatile emission from foliage have not been well documented.

JA is known to regulate a diverse array of plant responses, many of which relate to known and putative inducible defenses against biotic agents (Creelman and Mullet 1997). JA is an important signal in the production of many non-volatile defenses including protease inhibitors (Farmer et al. 1992), alkaloids (Gundlach et al. 1992) and steroids (Schmelz et al. 1998). Both mechanical damage (Creelman et al. 1992) and insect herbivory (McCloud and Baldwin 1997) are known to stimulate the production of JA signals in plants. For non-volatile direct defenses, Baldwin et al. (1997) demonstrated positive relationships between the levels of mechanical damage, tissue JA levels, and subsequent nicotine accumulation in tobacco (*Nicotiana sylvestris*). In this same system, application of OS from *M. sexta* larvae to wounded leaves increased the magnitude of the JA burst above that caused by damage alone (McCloud and Baldwin 1997). Compared to the wealth of information regarding a role for JA in regulating non-volatile defense chemistry, little is known about the regulation of insect-induced volatile emission.

Implications of a role for JA in plant volatile emission come primarily from exogenous applications of JA to both excised (Boland et al. 1995; Dicke et al. 1999) and intact plants (Halitschke et al. 2000; Schmelz et al. 2001). Less clear is how the endogenous JA levels in leaves, induced by insect herbivory, actually relate to plant volatile release. Recently, Halitschke et al. (2001) demonstrated that the fatty acid-amino acid conjugates present in *M. sexta* OS are sufficient to stimulate an increase in both JA levels and volatile release of (E)-α-bergamotene in wild tobacco (*N. attenuata*). If JA functions as an intermediary between the stimulus of caterpillar herbivory and induced-volatile emission, then increases in JA levels are necessary prior to volatile emission. In our experiments, insect-induced JA levels either paralleled or preceded volatile emission in the two time-course trials. Early in both time-course trials, JA displayed greater relative increases when compared to volatile emissions. These patterns are temporally consistent with a signaling role for JA. Moreover, by examining JA levels during volatile emission, we advance the mechanistic understanding of insect-induced volatile emission by demonstrating direct positive relationships between JA levels and both sesquiterpene and indole volatile emission. Our findings provide the first demonstration of quantitative relationships between JA and

volatile emission and thus strongly support the hypothesis that jasmonates are important regulators of insect-induced plant volatile emission.

In addition to JA, the levels of numerous other plant hormones have been found to fluctuate following mechanical damage. Some examples include indole acetic acid (Thornburg and Li 1991), abscisic acid (Peña-Cortés et al. 1989) and ethylene (Kende 1993). Multiple forms of plant stress have long been known to influence ethylene production (Abeles 1973). More recently, O'Donnell et al. (1996) demonstrated that ethylene is a necessary component in the signal cascade leading to wound-induced protease inhibitor accumulation in tomato seedlings. Both positive and negative interactions have been described with ethylene mediating the outcome of induced-plant responses. For example, ethylene and JA act synergistically in the induction of pathogenesis related genes (Xu et al. 1994), protease inhibitors (O'Donnell et al. 1996), and defensin genes (Penninckx et al. 1998). However, in tobacco, *M. sexta* OS applied to wound sites stimulated increases in JA levels yet resulted in the reduced accumulation of nicotine compared to damaged plants (McCloud and Baldwin 1997). It is now believed that an accompanying ethylene burst, which follows *M. sexta* OS application and actual herbivory, suppresses the induction of nicotine yet does not alter the induced volatile emission, namely (E)-α-bergamotene (Kahl et al. 2000; Winz and Baldwin 2001).

In our time-course experiments, the potential influence of insect-induced ethylene on volatile emissions was unclear. During BAW herbivory, peak ethylene production, relative to the controls, occurred at the same time as maximum volatile emission. In contrast to increases in JA levels, ethylene production was not significantly altered by herbivory within the first 4 h. This suggested that if induced ethylene production interacts with plant volatile emissions, it must occur in the later stages of volatile regulation, act at very low concentrations, or act through changes in ethylene sensitivity. Using an inhibitor of ethylene action we demonstrated that 1-MCP pretreatment reduces BAW-induced ethylene production and volatile emission without altering typical BAW-induced JA levels. 1-MCP inhibits ethylene production by repressing the activity of ethylene biosynthetic enzymes such as 1-aminocyclopropane-1-carboxylate synthase and also corresponding mRNA accumulation (Mathooko et al. 2001). However, the influence of ethylene production or perception on the accumulation of other plant defense signals is also possible. O'Donnell et al. (1996) demonstrated that ethylene can act to regulate wound-induced levels of JA and likewise salicylic acid accumulation during pathogen infection. In our recent experiments, we find no evidence that the inhibition of ethylene perception with 1-MCP or addition of ethylene gas alters the level of JA accumulated during BAW herbivory. In 1-MCP-treated plants, the significant reduction in volatile emission without decrease in JA accumulation indicates that ethylene perception also influences induced volatile emission.

Our research supports a role for jasmonates in regulating insect-induced volatile emission by demonstrating that increases in JA either precede or parallel increases in volatile emission and that quantitative relationships exist between insect-induced JA levels and volatiles. BAW herbivory also induces ethylene thus we examined the role of ethylene perception on induced volatile emission. Pretreatment of plants with an ethylene action inhibitor (1-MCP) decreased volatile emission without influencing insect-induced JA levels. Clearly, both JA levels and ethylene perception are important in the regulation of the insect-induced volatile emission in corn.

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